Ethylene Inhibitors Promote in vitro Regeneration of Medium Staple Cotton (Gossypium hirsutum L.) Cultivar Barac B-67

Eltayb Abdellatef and M.M. Khalafalla

Commission for Biotechnology and Genetic Engineering, National Centre for Research, Khartoum, Sudan

ABSTRACT

The promotive effects of ethylene inhibitors, silver nitrate (AgNO₃) and cobalt chloride (CoCl₂) on in vitro shoot regeneration from 35-day-old seedling-derived cotyledonary node explant of medium staple cotton (Gossypium hirsutum L. cv Barac B-67) was investigated. Shoot development was induced on cotyledonary node devoid of cotyledons and apical meristems cultured on B5 medium supplemented with kinetin (Kn) or benzyladenine (BA) alone or in combination. Both types and concentrations of cytokinins significantly influenced shoot proliferation. Kn proved to be more effective than BA. The efficiency of both BA and Kn for multiple shoot induction was positively affected when used in combination. The best response, however, was obtained when 2.5 mg/L Kn was used in combination with BA 0.1 mg/L. Addition of silver nitrate AgNO₃ and Cobalt chloride CoCl₂ to the medium enhanced regeneration frequency as well as number of shoots obtained per explant. The best result (3.2 shoot/explant) was obtained by using AgNO₃ at 3.0 mg/L. Elongation of multiple shoots was obtained on half strength agar-solidified B5 basal medium without plant growth regulators. More than 87% of the in vitro induced shoots produced roots when cultured on half strength B5 medium supplemented with 2.0% sucrose, 0.8% agar and 0.1 mg/L Naphthalene Acetic Acid (NAA). Rooted plants were hardened and 95% survived under greenhouse conditions.

Key words: In vitro regeneration, cotton, silver nitrate, cobalt chloride. G. hirsutum

Introduction

Decades of traditional breeding have been used in the improvement of agronomic traits of cotton (Gossypium). However, there remains of useful economic characters, including pest and disease resistance, stress tolerance and that may be amenable for further genetic enhancement of commercial cotton cultivars using modern breeding methods based on gene transfer technology.

The limiting step to the successful use of modern techniques in genetic improvement of the major crops has not been transgene insertion itself, but rather the regeneration of viable plants from the transgenic explant material (Murphy, 2003). Therefore, the success of any cotton improvement programme using gene transfer technological tools such as biolistics and Agrobacterium-mediated transformation depends on the availability of plant regeneration systems that are genotype-independent, efficient and which do not yield somaclonal variant (Firoozabady et al., 1987; Gould and Cedeno, 1998; McCabe and Mattinell, 1993).

The composition of the culture medium and the gaseous environment are important factors in regeneration of shoots in vitro. In recent years there has been increasing evidence that the occurrence of morphogenesis in cultured plant cells may be associated with ethylene (Saha et al., 2007). Varying amounts of ethylene is released in culture vessels during in vitro regeneration (De Proft et al., 1985). However, the role of ethylene in plant cells and tissues grown in vitro is not well understood. The presence of ethylene was found to be important for shoot morphogenesis in rice callus (Adkins et al., 1990) embryogenesis from anther cultures of Hordeum vulgare (Cho and Kash, 1989).
and flower bud formation from thin-layer explants of *Nicotiana tabacum* (Smulders et al., 1990). In contrast, ethylene accumulation inhibits *in vitro* shoot regeneration in *Nicotiana* (Huxter et al., 1981), *Triticum* (Purnhauser et al., 1987), *Zea mays* (Songstad et al., 1988) and *Brassica* (Chi et al., 1991). It was also observed that the addition of certain chemicals such as cobalt chloride or silver nitrate to the culture medium can inhibit ethylene production or its function by blocking certain steps in the pathway (Pua and Chi, 1993) and increases *in vitro* regeneration in monocots (Purnhauser et al., 1987; Songstad et al., 1988) and dicots (Chi et al., 1991; Roustan et al., 1989). Considering these information, the objective of this work was to describe the effect of silver nitrate and cobalt chloride on the frequency of adventitious shoot regeneration and number of shoots per explant of *in vitro* culture of cotyledonary node of medium staple cotton cultivar Barac B-67.

**Material and methods**

**Plant material**

Seeds of medium staple cotton cultivar (Barac B - 67) used in this study were obtained from the Agricultural Research and Technology Corporation (ARTC), Wad Medani, Sudan

**Seeds delinting and surface sterilization**

Seeds were delinted by using concentrated commercial H₂SO₄ (100 ml/kg of seeds). The seeds were continuously stirred in H₂SO₄ by spatula for 1 minute then washed by continuously running tap water for another 1 minute followed by thorough washing in sterile distilled water to remove traces of surface adherent. Under laminar flow cabinet seeds were disinfected by soaking in mercuric chloride HgCl₂ 0.2% (w/v) for 15 mints with continuous shaking and finally washed for five times by sterilized distilled water.

**Seed germination and explant preparation**

After surface sterilization 100 seeds were transferred to culture bottle and directly inoculated on the B5 (Gamborg et al., 1968) basal media and incubated for germination at 25°C±2 with a 16 h photoperiod. Cotyledonary nodes were removed from 35days-old *in vitro* raised seedlings. The cotyledons and apical meristem were excised and discarded. Thus, each explant had two dormant axillary buds. These decapitated cotyledonary nodes were used as explants throughout this study.

**Effect of cytokinins on *in vitro* morphogenesis**

Different concentrations (0.1, 0.5, 1.0, 2.5, or 5.0 mg/L) of BA and Kn alone or in combinations were added to the culture bottles containing B5 basal media in order to assess their effect on *In vitro* morphogenetic responses of the cotyledonary node.

**Effect of ethylene inhibitors on *in vitro* morphogenesis**

To determine the effects of ethylene inhibitors, B5 basal media containing Kn at 2.5 mg/L and BA 0.1 mg/L was supplemented with silver nitrate (AgNO₃) and cobalt chloride (CoCl₂) at different concentrations (1, 2, 3, 4, 5, 10 mg/L).

**Rooting of *in vitro* produced shoots**

Shoots (2-3 cm) derived from shoot bunches of cotyledonary node were excised and rooted on medium consisting of B5 and ½ B5 basal medium supplemented with 0.1,0.5,1.0 mg/L Naphthalene Acetic Acid (NAA) either Indole Acetic Acid (IAA) or Indole Butric Acid (IBA).

**Culture condition and Data analysis**

Cultures were incubated for six weeks at 25°C±2 with a 16 h photoperiod. All the media used in this study were supplemented with 2% (w/v) sucrose, solidified with 0.8% (w/v) and the pH was adjusted to 5.5 before addition of the agar and autoclaving at 121°C and 15 lb psi for 15 min.

Results were observed at regular intervals and data were collected from three independent experiments and presented as average ± standard error (SE).
Results and discussion

Cotton seeds obtained from the field are highly contaminated as they are covered with large numbers of small hairs that can hold spores of fungi and bacteria. Therefore in this study cotton seeds were firstly delinted by using concentrated H₂SO₄ then sterilized by HgCl₂ before in vitro germination. Delinting with H₂SO₄ is a highly effective way to remove the hairs and reduce the risk of contamination in the cultures. Disinfection of seeds through delinting with concentrated H₂SO₄ and then followed by HgCl₂ has already been proved to be essential in cotton tissue culture (Abdellatef and Khalafalla, 2007; Abdellatef and Khalafalla, 2008; Rauf et al., 2004).

Direct shoot bud differentiation was observed after 2 weeks of culture initiation. Multiple shoots were initiated from the cotyledonal node explants after 4 weeks of culture (Fig.1b). The frequency of shoot formation was influenced by types and concentrations of cytokinins used (Table1). Explants obtained from 35-days old seedling cultured on hormone-free B5 basal medium failed to show any response but remained green up to four weeks. However, on B5 basal medium supplemented with various concentrations of BA or Kn alone or in combinations enlarged in their size after one to two weeks of culture and adventitious shoots developed directly in another four weeks (Table1). These results agree with the finding of Jorge et al., (1998) who found that cytokinin is directly responsible for reprogramming the embryonic apical meristem axes of cotton towards the multiplication of buds.

Generally determination of optimal concentrations and combinations of growth regulators is necessary because unfavorable concentration may inhibit the growth of cellular mass as reported by Moore, (1998). Furthermore, the dose of cytokinin is known to be critical in multiple shoots induction (Abdellatef and Khalafalla, 2007). Therefore, in this study we compared the effects of various concentrations of BA and Kn on multiple shoots induced on cotyledonal node explants. The result showed that Kn at different concentrations induced more shoots per explant compared to BA at the same concentrations (Table 1). Indicating that, Kn was more efficient than BA for multiple shoot production from cotton cotyledonal node explant. Similar result was reported by Abdellatef and Khalafalla, 2007. The shoot regeneration frequency increased with increases of Kn concentration until it reaches 2.5 mg/L, which was found to be the optimal concentration for maximum frequency of shoot bud formation (2.6 shoots per explant). However, at higher concentration of both cytokinins the number of shoot per explant was reduced (Table 1). Zapata et al., (1999) had a similar observation with cotton shoot apices where plant regeneration was suppressed with higher concentrations of BA. This is mainly, because at higher cytokinin level cotyledonal node explant produced excessive callus and failed to improve the efficiency of shoot multiplication. Thiem, (2003) reported that callus growth on explant usually interfere with the propagation process.

### Table 1: Effects of cytokinin and cytokinin combination on multiple shoots induction on cotyledonal node explants obtained from 35-day-old seedlings of medium staple cotton cultivar (Barac- B- 67)

<table>
<thead>
<tr>
<th>Cytokinin (mg/L)</th>
<th>BA</th>
<th>Kin</th>
<th>Reg. culture (%)</th>
<th>No shoot/ explant (mean ± SE)</th>
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<tbody>
<tr>
<td>0.1</td>
<td>-</td>
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<td>0</td>
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<tr>
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<td>2.3±0.0</td>
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<td>- 1.0</td>
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<td>2.6±0.1</td>
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<tr>
<td>- 5.0</td>
<td>75</td>
<td>1.8±0.1</td>
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Table 2: Effect of ethylene inhibitors (silver nitrate and cobalt chloride) on multiple shoots induction on cotyledonal node explants of medium staple cotton cultivar (Barac- B -67) cultured on shoot formation media

<table>
<thead>
<tr>
<th>Ethylene inhibitor (mg/L)</th>
<th>Regeneration %</th>
<th>No of shoot/Explant (Mean ± SE)</th>
</tr>
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<tbody>
<tr>
<td>AgNO₃</td>
<td>CoCl₂</td>
<td>100</td>
</tr>
<tr>
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</tr>
<tr>
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<td>100</td>
</tr>
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<td>3.0</td>
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</tr>
<tr>
<td>4.0</td>
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<td>100</td>
</tr>
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<td>5.0</td>
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</tr>
<tr>
<td>10.0</td>
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<td>100</td>
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<tr>
<td>0.0</td>
<td>AgNO₃ 3.0 mg/L</td>
<td>100</td>
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<tr>
<td>0.0</td>
<td>4.0</td>
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<tr>
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<td>5.0</td>
<td>100</td>
</tr>
<tr>
<td>0.0</td>
<td>10.0</td>
<td>100</td>
</tr>
</tbody>
</table>

Fig. 1: *In vitro* induction of multiple shoots and plant regeneration of medium staple cotton cultivar (Barac-B-67) A. *In vitro* germinated seedling and cotyledonal node explant obtained from 35 days - old – seedling. B. Multiple shoots bunches induced from cotyledonal node explants on shoot formation media supplemented with AgNO₃ 3.0 mg/L. C. *In vitro* rooted shoot on half -strength B5 basal medium supplemented with NAA (0.1 mg/L). D. *in vitro* regenerated cotton plants in established soil.

In this study the result showed that combinations of Kn and BA positively affected the multiplication rate of the *in vitro* induced shoot compared to that obtained with cytokinine alone (Table 1). Kn 2.5 mg/L in combination with BA 0.1 mg/L induced the maximum No of shoot (2.8 shoot/ explant). The synergistic action of a combination of two or more cytokinins has also been reported in cotyledonal nodes of *Eclipta alba* (Baskaran and Jayabalan, 2005). In *E. impensa*, shoot segments incubated on a medium supplemented with 0.25mM BAP and 2.5mM kinetin produced more healthy shoots and overall shoot quality was better than observed for BAP used alone (Bunn, 2005). However, the multiple shoots obtained on combination of cytokinins failed to elongate on the same medium. Based on previous study carried in our laboratory (in press), it was found that half strength B5 basal medium without plant growth regulators was the most suitable media for shoot elongation, therefore for elongation shoot produced on B5 basal medium supplemented with Kn 2.5 mg/L and BA 0.1 mg/L were transferred individually to culture bottles containing half strength B5 basal medium without plant growth regulators and supplemented with 2% sucrose and 0.8% agar for 15 – 20 days. Cytokinin has been reported to regenerate cotton plants with short and compact shoots (Banerjee, 2000). Moreover, as in this study, cytokinins have often been reported to stimulate shoot proliferation, while inhibiting shoot elongation (Brassard, 1996). The use of hormone-free medium for shoot elongation has already been reported for soybean (Kaneda *et al.*, 1997) and faba bean (Khalafalla and Hattori, 1999).
growth was observed, 95% of the plants survived and all were morphologically normal (Fig. 1d).

One week. After three weeks, glass bottles were removed and transferred to greenhouse and placed under shade until

Khalafalla, 2007; Agrawal

medium containing 0.1 mg/L NAA (Table 3) (Fig. 1c). The promotory effect of reducing the salt concentration of

IBA. Among the different concentrations of NAA, rooting of cotton shoots was higher (87%) on half-strength B5

medium and using of NAA on rooting of

than full strength. Also NAA gave better result for root induction compared to basal media without or with IAA or

Alleviated by the addition of various additives will promote the application of plant tissue culture technology in the area of

selection resistance and production of cotton artificial seeds.

Cytokinins are ethylene inducing plant hormones and known to increase ethylene production several folds in

many plants (Saha et al., 2007), at least partially through the increase in ACC synthase activity (Ables et al., 1992).

BA synergistically enhanced ethylene production in mungbean hypocotyls (Yoshi and Imaseki, 1982). Kn slightly

stimulated ethylene production by etiolated seedlings in several species including pea and mung bean (Saha et al.,

2007). Based on the above-mentioned evidences the effects of ethylene inhibitors on cotton multiple shoot induction

was addressed by adding different levels of silver nitrate and cobalt chloride to the culture media containing Kn at

2.5 mg/L and BA 0.1 mg/L. Our result showed that, the presence of ethylene inhibitors namely AgNO 3 (3.0 mg/L)
or CoCl 2 (1.0 mg/L) in the shoot regeneration medium (B5 0.1 mg/L BA, ??2.5 Kn mg/L) was found to be beneficial

as they significantly enhanced the percentage shoot regeneration and number of regenerated shoots per explant

(Fig. 1b) (Table 2). The interaction of ethylene with other plant growth regulators is highly complex and is still little

understood. Interestingly, there are many contrasting examples which show that the regulation of ethylene levels

in tissue cultures can have both positive and negative effects on

in vitro

morphogenesis and proliferative growth.

Ethylene was shown to inhibit shoot regeneration in callus cultures of sunflower and tobacco (Huxter et al., 1981). The

negative effect of ethylene on morphogenesis in maize callus culture was demonstrated (Songstad et al., 1988).

Moreover, as reported in several recalcitrant plant species such as Chinese cabbage (Chi et al., 1991), mustard

(Pua and Chi, 1993), rice (Adkins et al., 1993), and in eastern white cedar, axillary shoot elongation (Nour and Thorpe,

1994). The above contrasting results show that the role of ethylene in

in vitro

morphogenesis, perhaps, vary from species to species and thus needs to be examined with

each plant genotype. Keeping this in view, in the present study the effect of two ethylene inhibitors AgNO 3 and

CoCl 2 on adventitious shoot proliferation from cotyledon node was investigated. AgNO 3 is believed to inhibit

ethylene action by competing with ethylene for binding sites located predominantly at the intracellular membrane

(Beyer, 1976,). In the present study, although both the ethylene inhibitors promoted adventitious shoot regeneration

from cotyledon explants, AgNO 3 appeared to be more effective than CoCl 2. The stimulation of shoot morphogenesis

elicted by AgNO 3 or CoCl 2 is in agreement with other reports on

Brassica campestris

(Chi and Pua, 1989),

Capsicum annuum

(Hyde and Philips, 1996),

Manihot esculenta

(Zhang et al., 2003),

Triticum aestivum

and

Nicotiana plumbaginifolia

(Purnhauser et al., 1987).

For

in vitro

rooting of the regenerated shoots, half strength B5 basal medium was found to be more effective than full strength. Also NAA gave better result for root induction compared to basal media without or with IAA or

IBA. Among the different concentrations of NAA, rooting of cotton shoots was higher (87%) on half-strength B5

medium containing 0.1 mg/L NAA (Table 3) (Fig. 1c). The promotory effect of reducing the salt concentration of

basal medium and using of NAA on rooting of

in vitro

induced shoots was reported for cotton (Abdellatief and

Khalafalla, 2007; Agrawal et al., 1997).

For acclimatization, plant were removed from rooting medium and transferred to plastic pots containing

autoclaved soil and covered with glass bottle to maintain humidity and were kept under culture room conditions for a

one week. After three weeks, glass bottles were removed and transferred to greenhouse and placed under shade until

growth was observed, 95% of the plants survived and all were morphologically normal (Fig. 1d).

Conclusion

Development of an efficient tissue culture and plant regeneration protocol for elite Sudanese cotton cultivars is the

first step towards the application of transgenic technology to improve cotton breeding and is, thus, the

foundation of cotton biotechnology. Furthermore, the present finding of enhancement of multiple shoot induction

by the addition of various additives will promote the application of plant tissue culture technology in the area of

selection resistance and production of cotton artificial seeds.

Table 3: The Effect of auxin and basal media strength on rooting of

in vitro

derived shoots of medium staple cotton cultivar (Barac B-76) after

6 weeks of culture

<table>
<thead>
<tr>
<th>Auxin (mg/L)</th>
<th>Rooting %</th>
<th>No of roots/shoot (Mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAA IAA IBA</td>
<td>½ B5 Full B5</td>
<td>½ B5 Full B5</td>
</tr>
<tr>
<td>0.0 0.0 0.0</td>
<td>37.0 50 0.8±0.3 0.6±0.3</td>
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<tr>
<td>0.1 0.0 0.0</td>
<td>87.0 75 1.4±0.3 0.8±0.3</td>
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<tr>
<td>0.5 0.0 0.0</td>
<td>75.0 50 0.8±0.2 0.9±0.2</td>
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<tr>
<td>1.0 0.0 0.0</td>
<td>50.0 50 1.1±0.5 1.0±0.5</td>
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<td>37.0 37 0.9±0.5 0.9±0.4</td>
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<td>0.0 0.0 0.0</td>
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Acknowledgment

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References


Chi, G.L., E.C. Pua and C.J. Goh, 1991. Role of ethylene on de novo shoot regeneration cotyledony explants of


Kaneda, Y., Y. Tabei, S. Nishimura, K. Harada, T. Akihama and K. Kitamura, 1997. Combination of thidiazuron and basal media with low salt concentrations increases the frequency of shoot organogenesis


